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# Multiplex PCR for Typing Strains of *Toxoplasma gondii*

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**A multiplex PCR assay was designed for multilocus strain typing of *Toxoplasma gondii* based on length polymorphism of five microsatellite markers. Eight *T. gondii* strains already sequenced at these five markers were used as control isolates. This method is simple, rapid, reproducible, and adapted to a large set of isolates.**

*Toxoplasma gondii* is an obligate intracellular apicomplexan with a worldwide distribution. This parasite can infect virtually all warm-blooded animals and uses felids as its definitive hosts. In humans, prevalence is high, ranging between 15 and 85% of the world adult human population, depending on geographical location (6). In immunocompetent children and adults, the infection is usually asymptomatic or revealed in 10% of cases by cervical or occipital lymphadenopathy (14). However, much less frequently, in immunocompetent individuals severe acquired forms, such as chorioretinitis (3), or pulmonary forms (4) have also been described. When acquired congenitally (17) or as a consequence of reactivation in immunocompromised patients (13), toxoplasmosis can be life-threatening.

The population structure of *T. gondii* is of considerable medical importance for understanding epidemiological patterns as well as heterogeneity in disease manifestations or for developing new strategies for vaccination, treatment, or diagnosis (16). In the domestic cycle of *T. gondii* (between cats, humans, and peri-domestic and meat-producing animals, such as pigs and sheep), there is a highly clonal population structure comprised of three widespread genotypes referred to as type I, type II, and type III (5, 10). In areas where breeding is recent or not intensive (such as Brazil and Africa) and in the wild cycle of *Toxoplasma* (e.g., in the Amazonian forest of French Guiana, where the cycle occurs between wild felids and a highly diverse set of wild intermediate hosts), atypical and recombinant genotypes prevail because of more-frequent genetic exchanges and a higher genetic diversity (2). Atypical and recombinant strains can also be encountered in Europe or the United States, although very rarely.

In humans, type II largely predominates in congenital toxoplasmosis, at least in Europe, but type I, atypical and recombinant genotypes are more frequently associated with severe forms of toxoplasmosis (1). Similarly, these last genotypes have been associated with severe cases of acquired toxoplasmosis in immunocompetent patients (4) and with acquired ocular toxoplasmosis (9). This correlation between disease and *Toxoplasma* genotype may justify the development of a typing method that will allow physicians to more aggressively monitor and treat infections in these cases.

Genotyping of isolates should be simple, rapid, reproducible, adapted to large series, and also informative enough to detect the genotype diversity of the species. With *Toxoplasma*, the majority of studies are based on PCR-restriction fragment length polymorphism analysis of the *SAG2* gene as described primarily in reference 11. This method is rapid and easy to use, but technical problems, such as incomplete amplification of the *SAG2* gene (7) or incomplete digestion of the DNA by restriction enzymes (12), have been reported; these problems may limit interest in the method and the reproducibility of its results. Furthermore, a monolocus analysis cannot detect recombination events or atypical alleles. Multilocus studies by PCR-restriction fragment length polymorphism analysis have been performed (10) but require several PCR assays. The data generated by multilocus sequencing studies (12, 8, 15, 2) are essential to better understand the population structure of

TABLE 1. Microsatellite markers and PCR primers used for multiplex PCR

| Marker (chromosome) | Accession no. | Coding function[a] | Primer sequences[b] |
|---|---|---|---|
| *TUB2* (IX) | M20025, AY572562 to AY572604 | Beta-tubulin gene | (F) 5′ 6FAM-GTCCGGGTGTTCCTACAAAA 3′<br>(R) 5′ TTGGCCAAAGACGAAGTTGT 3′ |
| *W35* (II) | W35487, AY572605 to AY572647 | Unknown (EST) | (F) 5′ GGTTCACTGGATCTTCTCCAA 3′<br>(R) 5′ 6FAM-AATGAACGTCGCTTGTTTCC 3′ |
| *TgM-A* (X) | Y17507, AY572649 to AY572691 | Myosin A gene | (F) 5′ GGCGTCGACATGAGTTTCTC 3′<br>(R) 5′ HEX-TGGGCATGTAAATGTAGAGATG 3′ |
| *B18* (VII) | BM189462, AY572693 to AY572735 | Unknown (EST) | (F) 5′ 6FAM-TGGTCTTCACCCTTTCATCC 3′<br>(R) 5′ AGGGATAAGTTTCTTCACAACGA 3′ |
| *B17* (XII) | BM175053, AY572737 to AY572779 | Unknown (EST) | (F) 5′ AACAGACACCCGATGCCTAC 3′<br>(R) 5′ HEX-GGCAACAGGAGGTAGAGGAG 3′ |

[a] EST, expressed sequence tag.
[b] F, forward primer; R, reverse primer.

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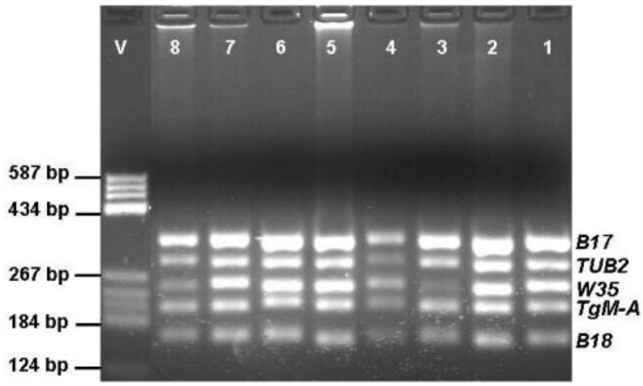


FIG. 1. Agarose gel electrophoresis of multiplex PCR amplification products with control isolates. Lanes: 1, BK; 2, ME49; 3, NED; 4, BOU; 5, DEG; 6, RMS-2001-MAU; 7, PSP-2003-KOM; 8, GUY-2002-MAT; V, molecular size markers.

*T. gondii* and to try to establish a link between genotype and clinical disease. However, sequencing is time-consuming and not adapted to an important set of isolates. In our previous study (2), we analyzed the nucleotide polymorphism in microsatellites (MS) and the flanking regions of five MS markers by sequencing. Because the majority of DNA polymorphism in these sequences was due to length polymorphism in the microsatellite region, we propose here a multiplex PCR able to detect these length polymorphisms by GeneScan analysis with only one PCR (instead of five by sequencing) in less than 1 day.

The five MS markers with a chromosome location and GenBank accession numbers corresponding to sequencing data of strains RH and 43 other isolates are listed in Table 1. Eight *T. gondii* control strains (BK, ME49, NED, BOU, DEG, RMS-2001-MAU, PSP-2003-KOM, and GUY-2002-MAT), already sequenced at the five MS markers, were used in this study (Table 2). *T. gondii* DNA was extracted from haploid stages (tachyzoites or bradyzoites) present in brains or ascitic fluids of infected mice using the QIAamp DNA mini kit (QIAGEN, Courtaboeuf, France). Five pairs of primers were designed for a multiplex assay (Table 1). In each pair, one primer was 5′-end labeled with fluorescein (6-carboxyfluorescein [6FAM] or hexachloro-6-carboxyfluorescein [HEX]) to allow sizing of PCR products with an automatic sequencer. Primers were synthesized by Applied Biosystems (Courtabœuf, France).

For PCR, we used the QIAGEN multiplex PCR kit with 2× QIAGEN multiplex PCR master mix (final concentration, 1×), a 0.04 μM concentration of each primer, 6 μl of distilled water, and 4 μl of DNA in a total volume of 25 μl. Amplifications were carried out in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems) using the multiplex PCR protocol for amplification of microsatellite loci (QIAGEN Multiplex PCR kit; QIAGEN): 15 min at 95°C (initial activation step), followed by 35 cycles consisting of 94°C for 30 s, 63°C for 3 min, and 72°C for 60 s. The last extension step was at 60°C for 30 min. A first electrophoresis of PCR products with molecular weight marker V (Roche Diagnostics, Meylan, France) was realized on a 2% agarose gel stained with ethidium bromide in order to confirm DNA amplification (Fig. 1). When bands are intense, such as in Fig. 1, PCR products have to be diluted 1/15 in deionized formamid before electrophoresis in an automatic

TABLE 2. Correlation between microsatellite sequencing data and length polymorphism results from multiplex PCR

| *Toxoplasma* strain (accession no.)[a] | MS type[a] | Microsatellite sequence (allele) | | | | | Multiplex length polymorphism (allele) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *TUB2* | *W35* | *TgM-A* | *B18* | *B17* | *TUB2* | *W35* | *TgM-A* | *B18* | *B17* |
| BK (AY572593, AY572621, AY572650, AY572714, AY572751) | I | $(CA)_8$ (1) | $(TC)_{10}(TG)_2$ (1) | $(TG)_9$ (1) | $(CA)_{10}$ (1.3) | $(TC)_{10}$ (1) | 291 (1) | 248 (1.6) | 204 (1) | 158 (1.3) | 340 (1) |
| ME49 (AY572581, AY572631, AY572661, AY572710, AY572759) | II | $(CA)_7$ (2.3) | $(TC)_7(TG)_2$ (2) | $(TG)_8$ (2) | $(CA)_9$ (2) | $(TC)_7$ (2.3) | 289 (2.3) | 242 (2.3.7) | 202 (2) | 156 (2) | 334 (2.3) |
| NED (AY572595, AY572632, AY572662, AY572726, AY572760) | III | $(CA)_7$ (2.3) | $(TC)_6(TG)_3$ (3) | $(TG)_7$ (3) | $(CA)_{10}$ (1.3) | $(TC)_7$ (2.3) | 289 (2.3) | 242 (2.3.7) | 200 (3) | 158 (1.3) | 334 (2.3) |
| BOU (AY572577, AY572643, AY572675, AY572731, AY572774) | II* | $(CA)_7$ (2.3) | $(TC)_8(TG)_2$ (5) | $(TG)_8$ (2) | $(CA)_9$ (2) | $(TC)_7$ (2.3) | 289 (2.3) | 244 (5) | 202 (2) | 156 (2) | 334 (2.3) |
| DEG (AY572583, AY572624, AY572653, AY572723, AY572769) | II | $(CA)_7$ (2.3) | $(TC)_7(TG)_2$ (2) | $(TG)_8$ (2) | $(CA)_9$ (2) | $(TC)_7$ (2.3) | 289 (2.3) | 242 (2.3.7) | 202 (2) | 153 (2)[b] | 334 (2.3) |
| RMS-2001-MAU (AY572591, AY572630, AY572660, AY572719, AY572758) | II/I* or II/III* | $(CA)_7$ (2.3) | $(TC)_7(TG)_2$ (2) | $(TG)_{10}$ (5) | $(CA)_{10}$ (1.3) | $(TC)_7$ (2.3) | 289 (2.3) | 242 (2.3.7) | 206 (5) | 158 (1.3) | 334 (2.3) |
| PSP-2003-KOM (AY572600, AY572642, AY572671, AY572728, AY572772) | I/III* | $(CA)_8$ (1) | $(TC)_9(TG)_3$ (6) | $(TG)_7$ (3) | $(CA)_{10}$ (1.3) | $(TC)_{10}$ (1) | 291 (1) | 248 (1.6) | 200 (3) | 158 (1.3) | 340 (1) |
| GUY-2002-MAT (AY572597, AY572629, AY572659, AY572721, AY572757) | Atypical | $(CA)_8$ (1) | $(TC)_6TT(TG)_2$ (7) | $(TG)_6$ (4) | $(CA)_{10}$ (1.3) | $(TC)_3TT(TC)_3$ (11) | 291 (1) | 242 (2.3.7) | 198 (4) | 158 (1.3) | 337 (11) |

[a] GenBank accession numbers and MS types are described in reference 2. *, one of the five alleles defining this MS type was an atypical allele not found in the classical type (as described in reference 2).
[b] Allele 2 is associated with a deletion of 3 bp in the microsatellite flanking region.

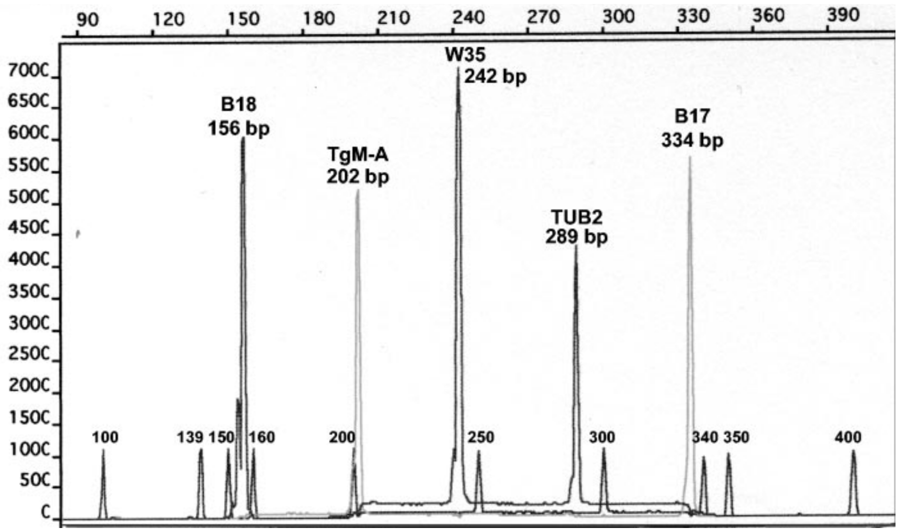


FIG. 2. Electrophoresis of multiplex PCR amplification products from a type II isolate (ME49) with an automatic sequencer by using GeneScan software. The *x* axis indicates a size fragment scale (in base pairs). The *y* axis indicates a peak height scale corresponding to the fluorescence intensity of detected peaks. Size fragments of GeneScan size standard ROX 500 are indicated between 75 and 500 bp.

sequencer. One microliter of each diluted PCR product was mixed with 0.5 μl of the red-dye-labeled GeneScan size standard ROX 500 (Applied Biosystems) and 23 μl of deionized formamid. This mixture was then denatured and run on a polyacrylamide gel POP4 (Applied Biosystems) in a 47-cm by 50-μm capillary for genetic analysis POP4 (Applied Biosystems). Signals were read with an automatic sequencer (Abiprism 310 collection 1.0; Applied Biosystems), and the data were stored and analyzed with GeneScan analysis software (version 2.1; Applied Biosystems) (Fig. 2).

Correlation between microsatellite sequence and length polymorphism results from the Multiplex assay are reported in Table 2. MS polymorphism consists mainly in size variation of dinucleotide tandem repeats. This length polymorphism of microsatellite regions can be assessed with fluorescent primers after electrophoresis on an automatic sequencer. For instance, at *TgM-A*, the differences between allele 1 $(TG)_9$, allele 2 $(TG)_8$, or allele 3 $(TG)_7$ are detected by our Multiplex assay with 204, 202, and 200 bp, respectively. Atypical alleles at *TgM-A*, such as $(TG)_6$ or $(TG)_{10}$, are also detected with 198 and 206 bp, respectively. Thus, the multilocus genotype results of multiplex PCR with five MS markers can easily distinguish the various genotypes used in this study: type I (BK), type II (ME49), type III (NED), a type II-related genotype (BOU), and recombinant (RMS-2001-MAU and PSP-2003-KOM) or atypical (GUY-2002-MAT) genotypes.

However, not all the genetic polymorphisms of microsatellite regions shown by sequencing can be detected by this multiplex assay because of different combinations of dinucleotide repeats or single-nucleotide polymorphisms in the microsatellite sequence. This is mainly the case for the *W35* microsatellite sequence, which is made up of two different dinucleotide repeats. For instance, the 242-bp length at *W35* by multiplex PCR corresponds to three different sequences: $(TC)_7(TG)_2$, which is allele 2, $(TC)_6(TG)_3$, which is allele 3, and $(TC)_6TT(TG)_2$, which is an atypical allele 7. Similarly, the 248-bp length at *W35* corresponds either to $(TC)_{10}(TG)_2$, which is allele 1, or to $(TC)_9(TG)_3$, which is another atypical allele (allele 6). However, the association with the four other MS markers allows us to distinguish type I, II, or III or an atypical genotype. On the other hand, all length polymorphism may not be due to microsatellite length polymorphism but to rare insertions or deletions in flanking sequences which are detected by our multiplex assay; for instance, strain DEG has allele 2 at *B18*, which is $(CA)_9$ and should be detected at 156 bp, but because of a deletion of 3 bp in flanking regions, the detected length is 153 bp. An insertion of 3 bp in GUY-2002-MAT explains the unusual length of 337 bp at *B17* with the multiplex assay. In these insertion or deletion events, only sequencing can affiliate the correct MS allele.

In conclusion, we have developed a multiplex PCR for *Toxoplasma* strain typing which is (i) simple, as only one PCR is needed to perform multilocus typing with five markers; (ii) rapid, as the typing results can be available in 1 day; and (iii) reproducible and adapted to large series (the reproducibility of the multiplex PCR assay has been tested in a large series of isolates from 200 human patients with congenital cases collected in France by the different laboratories of the French parasitologist network for *Toxoplasma* isolate collection or the BRC ToxoBS group [see Acknowledgments]). The known overrepresentation (84.88% of strains) of only one multilocus genotype (type II) in human patients with congenital disease in France (1) has been confirmed with this multiplex assay (data available from the authors).

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## REFERENCES

1. **Ajzenberg, D., N. Cogné, L. Paris, M. H. Bessieres, P. Thulliez, D. Filisetti, H. Pelloux, P. Marty, and M. L. Dardé.** 2002. Genotype of 86 *Toxoplasma gondii* isolates associated with human congenital toxoplasmosis, and correlation with clinical findings. J. Infect. Dis. **186:**684–689.
2. **Ajzenberg, D., A. L. Bañuls, C. Su, A. Dumètre, M. Demar, B. Carme, and M. L. Dardé.** 2004. Genetic diversity, clonality and sexuality in *Toxoplasma gondii.* Int. J. Parasitol. **34:**1185–1196.
3. **Burnett, A. J., S. G. Short, J. Isaac-Renton, A. King, D. Werker, and W. R. Bowie.** 1998. Multiple cases of acquired toxoplasmosis retinitis presenting in an outbreak. Ophthalmology **105:**1032–1037.
4. **Carme, B., F. Bissuel, D. Ajzenberg, R. Bouyne, C. Aznar, M. Demar, S. Bichat, D. Louvel, A. M. Bourbigot, C. Peneau, P. Neron, and M. L. Dardé.** 2002. Severe acquired toxoplasmosis in immunocompetent adult patients in French Guiana. J. Clin. Microbiol. **40:**4037–4044.
5. **Dardé, M. L., B. Bouteille, and M. Pestre-Alexandre.** 1988. Isoenzymic characterization of seven isolates of *Toxoplasma gondii* by isoelectrofocusing in polyacrylamide gels. Am. J. Trop. Med. Hyg. **39:**551–558.
6. **Dubey, J. P., and C. P. Beattie.** 1988. Toxoplasmosis of animals and man. CRC press, Inc., Boca Raton, Fla.
7. **Fuentes, I., J. M. Rubio, C. Ramírez, and J. Alvar.** 2001. Genotypic characterization of *Toxoplasma gondii* strains associated with human toxoplasmosis in Spain: direct analysis from clinical samples. J. Clin. Microbiol. **39:**1566–1570.
8. **Grigg, M. E., S. Bonnefoy, A. B. Hehl, Y. Suzuki, and J. C. Boothroyd.** 2001. Success and virulence in *Toxoplasma* as the result of sexual recombination between two distinct ancestries. Science **294:**161–165.
9. **Grigg, M. E., J. Ganatra, J. C. Boothroyd, and T. P. Margolis.** 2001. Unusual abundance of atypical isolates associated with human ocular toxoplasmosis. J. Infect. Dis. **184:**633–639.
10. **Howe, D. K., and L. D. Sibley.** 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J. Infect. Dis. **172:**1561–1566.
11. **Howe, D. K., S. Honoré, F. Derouin, and L. D. Sibley.** 1997. Determination of genotypes of *Toxoplasma gondii* isolates isolated from patients with toxoplasmosis. J. Clin. Microbiol. **35:**1411–1414.
12. **Lehmann, T., C. R. Blackston, S. F. Parmley, J. S. Remington, and J. P. Dubey.** 2000. Strain typing of *Toxoplasma gondii*: comparison of antigen-coding and housekeeping genes. J. Parasitol. **86:**960–971.
13. **Luft, B. J., and J. S. Remington.** 1992. Toxoplasmic encephalitis in AIDS. Clin. Infect. Dis. **15:**211–222.
14. **Montoya, J. G., and O. Liesenfeld.** 2004. Toxoplasmosis. Lancet **363:**1965–1976.
15. **Su, C., D. Evans, R. H. Cole, J. C. Kissinger, J. W. Ajioka, and L. D. Sibley.** 2003. Recent expansion of *Toxoplasma* through enhanced oral transmission. Science **299:**414–416.
16. **Tibayrenc, M., F. Kjellberg, J. Arnaud, B. Oury, S. F. Brenière, M. L. Dardé, and F. J. Ayala.** 1991. Are eukaryotic microorganisms clonal or sexual? A population genetics vantage. Proc. Natl. Acad. Sci. USA **88:**5129–5133.
17. **Wong, S. Y., and J. S. Remington.** 1994. Toxoplasmosis in pregnancy. Clin. Infect. Dis. **18:**853–861.